

Mouse model with age-dependent immune response and immune-tolerance for HBV infection

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ABSTRACT

Objective: Viral clearance of human HBV infection largely depends on the age of exposure, so a mouse model with age-dependent immune response and immune-tolerance for HBV infection is essential.

Methods: HBVRag1 mice were generated by crossing Rag1^{-/-} mice with HBV-Tg mice. The differences between adult and young HBVRag1 mice were detected after adoptive transfer of splenocytes. Immune tolerance was evaluated by quantitative hepatitis B core antibody (qAnti-HBc) assay, adoptive transfer, and modulation of gut microbiota with antibiotic.

Results: After HBVRag1 mouse reconstitution, adult mice showed obvious HBV-dependent inflammation and hepatocytes damage, cleared HBsAg and generated HBsAb and HBcAb, but young mice never developed ALT elevation, and only generated HBcAb with persistence of HBsAg. In addition, for adult mice, more hepatic CD8⁺T and B cells promoted clearance of HBsAg 30 days after lymphocytes transfer, and for young mice, higher levels of cytokines link to

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the persistence of viral antigens during initiation of immune response towards HBV. The level of qAnti-HBc increased significantly with the time of adoptive transfer in young mice, but decreased significantly in adult within our model. This mimics kinetic changes of human HBV infection regarding qAnti-HBc level. Also, the age-related tolerance in this model was different from which was in HBV-Tg mice, and can be regulated through modulation of gut microbiota. Meanwhile, GS-9620 can achieve inhibition of HBsAg, but HBV vaccine just clears limited HBsAg within the model.

Conclusions: Here, we described a mouse model with age-dependent immune response and immune tolerance of HBV infection which could mimic chronic HBV infection in human. It will open a door for evaluating new therapeutic approaches before clinical trials.

Keywords: HBV transgenic mice; Mouse model; Immune tolerance; age; GS-9620; Therapeutic vaccination

1 Introduction

There are still about 400 million people chronically infected with hepatitis B virus and one million deaths annually worldwide [1, 2]. The unique feature of viral clearance of HBV infection within human is age-dependent. After HBV infections 95% of adult will be achieve spontaneous clearance while 90% of neonates will develop chronic infection [2]. “Immune system immaturity”, “neonatal tolerance” or “liver tolerance” may result in chronic HBV infection in the young presumably [2, 3]. A diverse and strong adaptive immune response is necessary for viral clearance in the adult [4]. But why the disease outcomes change with the age of infection remains unclear. Early events of immune response which determine HBV clearance or persistence should be taken seriously [5-7].

Since HBV infection is restricted to humans, development of animal models has also been the biggest challenge of knowing the mechanism of chronic HBV infection. In a suitable model, persistent HBV replication and expression need to be established, HBV-specific adaptive immune responses must be induced [8]. In order to overcome this difficulty and investigate the immunological mechanisms of age-dependent viral clearance during HBV infection, Baron JL group set up model systems with C57 background mouse, which can mimic human HBV clearance and persistence [6, 9]. IL-21 and CXCL13 expressed by T follicular helper cells and macrophages respectively in the liver promoted hepatic lymphoid organization and successfully induced immunity against HBV infection.

Accumulated evidences demonstrate that host genetic factors determine chronic HBV infection [3]. Although age-dependent viral clearance of HBV infection in the immune reconstitution mouse model was not affected by genetic background [5, 6], it varied significantly in the HDI (hydrodynamic injection) mouse model [3]. For instance, age-dependent viral clearance was not found in BALB/c background HDI mouse model [3]. Therefore, to investigate age-dependent HBV clearance in BALB/c mice in immune reconstitution mouse model is essential.

In this study, we obtained BALB/c background HBVRag1 mice by crossing HBV-Tg mice with Rag1^{-/-} mice. The HBVRag1 mice expressed HBsAg and HBcAg without adaptive immune system. Adoptive transfer of splenocytes from BALB/c mice (which were naive to HBV), mimics the primary HBV infection, and reveals age-dependent viral clearance and immune responses. The immune tolerance in young mice was similar to chronic HBV infection in human, and can mimic host anti-HBV immunity in kinetic changes of qAnti-HBc level. We try to look

into immunotherapy of GS-9620 and HBV vaccine within the model.

2 Material and methods

2.1

Mice and experimental system

BALB/c background HBV-Tg mice were produced in our center by 1.3 copies HBV (ayw subtype) genome, which has detectable HBV DNA and high level of HBsAg [10-12]. BALB/c background Rag1^{-/-} mice (Strain Name: C.129S7 (B6)-Rag-1^{tm1Mom}/J) provided by Dr. Shengdian Wang of Institute of Biophysics, CAS, were purchased from the Jackson Laboratory. BALB/c mouse was from Southern Medical University (Guangzhou, China). BALB/c background HBVRag1 mice were produced using HBV-Tg mice crossed to Rag1^{-/-}. Young (3 weeks of age) and adult (8-9 weeks of age) HBVRag1 mice were given 5×10^7 syngeneic splenocytes through tail vein injection. The ALT detection Kit was from Jiancheng Co., (Nanjing, China). All mice were kept under the specific pathogen-free condition at No.458 Hospital (Guangzhou, China) following institutional guidelines.

2.2

PCR analysis integrated transgenes

Genomic DNA was completely isolated from the 2-week-old mice tail samples (2~3mm) as described in protocol of E.Z.N.A. Tissue DNA kit (OMEGA bio-tek). DNA was amplified with primers of HBV DNA, P1: 5-ACGCAGGATAACCACATT-3, P2: 5-ACAACCTTCCACCAAAC-3, while analyzing Rag1^{-/-} mice genomic DNA with P3: 5-TGGATGTGGAATGTGTGCGAG-3, P4: 5-GAGGTTCCGCTACGACTCTG-3, P5: 5-CCGGACAAGTTTTTCATCGT-3 [13, 14].

2.3 HBV protein and antibody detections

First, we detect serum from mice for HBsAg and HBeAg with ELISA kit (Kehua, Shanghai, China), and HBsAb was assayed with ETI-AB-AUK PLUS and ABAU set (DiaSorin, Italy, sensitivity 5 mIU/ml). Then, serum from transfer HBVRag1 mice was detected for HBcAb with ETI-AB-COREK PLUS (DiaSorin, Italy), and for the quantitative HBcAb detection with double sandwich immunoassay (dynamic range 0.08–2.5 IU/ml; Wantai, Beijing, China). Samples were tested at dilutions of 1:10 to 1:40 if the Anti-HBc level > 2.5 IU/mL. All assays were read on Multiskan FC (Thermo SCIENTIFIC).

2.4 Liver lymphocytes preparations

Briefly, HBVRag1 mice were perfused with 20 ml cold PBS (pH7.2), until livers became swollen and blanched. Then livers were removed from abdomen and forced through 75 μ m nylon film filter. After 60-second centrifuged at 35 g, supernatant was collected and centrifuged again with 550 g for 8 minutes. The pellet was resuspended in PBS and liver-derived lymphocytes were isolated with 70/40 percoll [6, 15].

2.5

ELISpot assay

2×10^5 liver splenocytes/well was placed on 96-well plates with ELISpot assays for IL-4 and IFN- γ (Dakewe, Shenzhen, China). Cells were unstimulated for 21 h at 37 °C while positive control was stimulated with 2 g/ml PMA/Ionomycin. After washing and staining, spot forming cells/well (SFC) were counted by ELISpot Reader Champ SpotII.

2.6

Flow cytometry

Lymphocytes were stained according to the protocols (the following antibodies were from BD Pharmingen, USA): APC-conjugated anti-IgM (II/41), PE-conjugated anti-CD45R/B220

(clone RA3-6B2), PE-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD8a (clone 53-6.7), and PE-CyTM conjugated anti-CD3e (clone 145-2C11). Cells were analyzed with a FACS Calibur flow cytometer (BD) and CellQuest software (BD)

2.7

Liver histology

Tissues were fixed with 10% formalin and embedded in paraffin. Sections with the thickness of 5-micron were stained with hematoxylin and eosin (HE) according.

Immunohistochemistry was performed using goat anti-HBsAg (Abcam ab17183) or rabbit anti-HBcAg (GB058602) by the Microscopy (OLYMPUS BX43F).

2.8

Antiviral effect of GS-9620 and HBV vaccine in mouse model.

The young HBVRag1 mice (3 weeks old) were reconstituted with syngeneic splenocytes and were named as age-dependent young (ADY) mouse model of HBV infection after 6 weeks of adoptive transfer. (1) GS-9620 treatment: GS-9620 from MedChem Express of Shanghai with purity > 98%. Mice were treated by gavage with GS-9620 at 5 mg/kg body weight 3 times a week for 4 weeks. (2) HBV vaccine treatment: recombinant yeast HBsAg vaccine (subtype: adw) was provided by BioKangtai Company of Shenzhen. Mice were vaccinated with 5 µg HBV vaccine and boosted on day 14. The mice were bled through the tail vein at regular intervals, and HBsAg and anti-HBsAg were analyzed after serum was isolated.

2.9

Statistical analysis

All categorical data were analyzed using the Fisher's exact test, or Mann-Whitney U test, as appropriate, P values < 0.05 were considered statistically significant. All analyses were

performed by SPSS13.0.

3 Results

3.1

Production and characterization of the HBVRag1 mice

14 offspring mice were produced after 3 female HBV-Tg mice were crossed with 3 male Rag1^{-/-} mice. 12 mice were found to have HBV and heterozygote Rag-1 (rag1^{+/-}) gene after PCR detection (Fig. 1A), and among which HBsAg were detected in 10 mice, and Then backcross the heterozygote Rag1^{+/-} mice with Rag1^{-/-} mice, and Rag1^{-/-} mice which could express HBsAg in the serum were finally selected. These mice expressed HBsAg in the plasma and were found to have no mature B or T cell in the spleen and liver by flow cytometry analysis, were finally named as HBVRag1 mice.

In the serum of HBVRag1 mice, no HBeAg or anti-HBs detected, but HBsAg existed. The level of HBsAg significantly enhanced during 3 weeks to 5 weeks old ($P < 0.01$), and no fluctuation difference showed during the lifespan. The expression of HBsAg did not differ by sex of the mice (from 3.5 weeks of age to month 28) ($P > 0.05$). And it expressed in the cytoplasm of most hepatocytes, while HBcAg was detected in almost all the hepatocytes nucleus, but minorly in the cytoplasm (Fig. 1B/C).

3.2

Appropriate number of adult splenocytes for adoptive transfer

To settle number of spleen cells for reconstitution of HBVRag1 mice, 10^8 , 10^7 , 10^6 , 10^5 and 10^4 of splenocytes from adult wild-type BALB/c mice were transferred to adult HBVRag1 mice respectively. Only in group of 10^8 and 10^7 did the level of HBsAg declined significantly after adoptive transfer, as well as HBsAb was found 4 weeks later (Fig. 2A/B). But for HBcAb

production, all of groups have been detected to be positive 4 weeks after adoptive transfer except for the 10^4 group (Fig. 2C). The production of HBcAb showed a dose-dependent correlation with the amount of spleen cells transferred. Taken together, there is no difference in HBsAg decline and production of HBcAb in both groups 10^8 and 10^7 , in the following experiment 5×10^7 wild-type adult splenocytes were adoptive transfer to HBVRag1 mice for reconstitution.

3.3

Adult mice generated HBsAb and HBcAb, clearing HBsAg with more CD8 T and B cell, while young mice only produced HBcAb and more cytokines during primary HBV infection after adoptive transfer

After receiving naive adult splenocytes from BALB/c mice, adult HBVRag1 mice developed liver injury rapidly. There were two peaks of ALT rise, on day 7 and day 21 respectively (Fig.3A). Obviously, there were lobular inflammation and hepatic injury (Fig.3B). However, young mice never revealed ALT rise or hepatocellular damage (Fig.3A/C).

Interestingly, the level of IFN- γ and IL4 from hepatic lymphocytes of young HBVRag1 mice eight days after adoptive transfer is higher than that in adult (Fig.3D), which implies that in primary stage of immune response to HBV, the high level of cytokines in young mice may lead to viral persistence. Whether IFN- γ plays a tolerogenic role or is helpful in eliciting effective anti-HBV immune response during early infection in young mice liver need to be carefully investigated [16, 17].

The clearance of HBsAg from circulation (Fig.4A) and production of HBsAb and HBcAb were observed in adult HBVRag1 mice following adoptive transfer (Fig.4B and C). But young mice failed to produce HBsAb, and transient clearance of serum HBsAg was showed only in a small number of mice (Fig.4A and B). Meanwhile, HBcAb production was consistent with that

of adult mice, and HBcAb was produced in all of the young mice from 7 days after adoptive transfer (Fig.4C). Consider together, while adult mice generated HBsAb and HBcAb and cleared HBsAg from circulation, young mice only generated HBcAb with persistence of HBsAg. The age-dependent immune responses in adult and young HBVRag1 mice are similar to those observed in adult and young humans. This primary HBV infection mouse model reflects the vital differences in viral clearance between young and adult human.

In addition, comparing with young mice, there was a significant, early increase in the percentages of T cells, CD4⁺ T cells, but not in CD8⁺ T cells or B cells in the livers of adult mice eight days following adoptive transfer. However, 30 days later, the proportion of CD8⁺ T and B cells significantly elevated (Fig.4D and E). The results indicated that CD8⁺ T cells and B cells were pivotal in the clearance of HBsAg in adult mice. Moreover, the delayed vigorous expansion of CD8⁺ T cell and B cell in adult mice indicated conversely that the initial T cell expansion was similar between young and adult mice. Consistent with this finding, the initial T cell expansion was also similar in the natural LCMV infection in mice [18].

3.4

The age-related tolerance in ADY model mimics human chronic HBV infection, which is

different from what it is in HBV-Tg mice, and can be regulated by gut microbiota

The mechanism of HBV-induced immune tolerance remains unclear. And it has been controversial that whether HBV-Tg mice could mimic immune tolerance of patients with chronic hepatitis B [8, 19]. Serum quantitative hepatitis B core antibody (qAnti-HBc) level is determined by host immune status, and strongly associated with hepatitis activity and HBeAg seroconversion in CHB patients [20-22]. Our HBV-Tg mouse has low level of qAnti-HBc [23],

which increased little when aging (Fig.5A). But ADY mouse model had significantly higher ($P < 0.01$, approximately 10-fold) serum qAnti-HBc level than HBV-Tg mice (Fig.5A and B), which was similar to the phase of immune clearance (IC) and immune tolerance (IT) in human HBV infection [21]. Additionally, the level of qAnti-HBc in adult HBVRag1 mice was significantly higher than that in young mice 14 days after adoptive transfer (Fig.5B). However, the level of qAnti-HBc gradually decreased in adult mice with the clearance of HBsAg, but increased in young mice with the persistence of HBsAg (Fig.5B). The kinetic change of qAnti-HBc indicates that anti-HBV immune response which is persistent in young mice is similar to the natural history of HBV infection in human, when chronic hepatitis smoldering leads to gradual increase of HBcAb [21].

Furthermore, in order to further compare the differences in immune tolerance between ADY mice and HBV-Tg mice, we transferred the splenocytes from those mice to HBVRag1 mice accordingly [24]. The level of HBsAg in recipient HBVRag1 mice decreased significantly within 2 weeks in ADY mouse model group, but there's no change in HBV-Tg mice group (Fig.5C). The results indicated that HBV-Tg mice were more tolerant to HBV than ADY mouse model.

In fact, the age-related immune tolerance could be regulated in HDI mouse model by antibiotic treatment for that gut microbiota could regulate liver innate immunity pathway [3]. Here, we found that after treating HBVRag1 mice with water contained ampicillin from 3 weeks to 8 weeks of age, adult mice were also found tolerant to HBV exposure after adoptive transfer, which was similar to young counterparts (Fig.5D). The age-related HBsAg clearance disappeared in most of ampicillin treated adult mice. The results imply that ampicillin treatment may affect the liver innate immunity pathway through the gut–liver axis [3, 25]. In conclusion, this age-

related tolerance in ADY model was different what it is in HBV-Tg mice, and can be regulated by gut microbiota, which could mimic human HBV infection in kinetic changes of qAnti-HBc level.

3.5

Activity of GS-9620 and HBV vaccine in ADY mouse model

GS-9620 being an oral TLR7 agonist has anti-HBV effect in woodchuck [26], chimpanzees [27], and is well tolerated in humans [28]. Orally treated GS-9620 5 mg/kg body weight 3 times a week for 4 weeks, comparing with control group in ADY mouse model, the level of HBsAg was found to have decreased significantly from the 2nd-week in GS-9620 treated group (Fig.6A).

The recombinant hepatitis B vaccine has specific but limited impact on chronic hepatitis B patients [8, 29]. But Tsuge M [30] reported antiviral effects of HBIG and HBV vaccine on HBsAg seroclearance in CHB patients whose HBsAg level was less than 500 IU/ml. Hereby we found that, HBV vaccine could induce sustained seroconversion in most of ADY model mice with high level HBsAb (120 ± 248 mIU/ml) when HBsAg below 1000 COI (Fig.6B), while systemic immune tolerance was shown towards HBsAg with no anti-HBs detected when HBsAg level being over 1000 COI (Fig.6C).

4 Discussion

Herein, we confirmed that the age of hepatic microenvironment in BALB/c genetic background HBVRag1 mice obviously affected viral clearance. After receiving an adult immune system, young mouse produced only HBcAb, along with few hepatic CD8⁺ T and B cells, and failed to produce HBsAb with HBsAg persistence, thus mimics chronic HBV infection in young human. Differences of immune responses between young and adult mice were similar to that in

C57 background mouse model [6]. However, HBsAg in a few young mice was cleared transiently with no anti-HBs detected three weeks after adoptive transfer. In all, the mechanism of transient HBsAg clearance in young mice should be discussed.

In an immunodeficient mouse model, a naive immune system being suddenly exposed to large amount of virion would affect the development of immune cells [6]. Our mouse model was based on immunodeficient HBV-Tg mice (HBVRag1), but the high level of cytokine during the initiation of an immune response in young mice was similar to which was observed in natural LCMV infected subjects [18]. Moreover, the kinetic change of qAnti-HBc level associating with host anti-HBV immunity in our mouse model was also similar to natural human HBV infection. However, our mouse model was not naturally HBV infected and it is expected that humanized mice would open a door for us to study natural HBV infection [31].

Whether the immune tolerance of a mouse model could mimic human HBV infection is very important. Both functional and deletional tolerance might be effective in preventing the clearance of human HBV infection [32] and the liver has the function of promoting immune tolerance rather than immunity [33]. However, among the widely used two mouse models, the immune tolerance in HBV-Tg mice was considered central clonal deletion of HBV-specific T and B cells [34], while functional tolerance in HDI mouse model was considered to be mediated by Tr1-like cells, which was inconsistent with chronic hepatitis B patient [24, 35]. But which kind of cells and molecules mediated age-related tolerance in this mouse model is worthy of further study.

Functional cure of CHB by bone marrow transplant strongly recommended that repriming an invalid immune response may resolve chronic HBV infection [36]. But therapeutic

vaccination for CHB has only limited efficiency so far [29, 37]. Immune tolerance difference between mouse model and CHB patients may be one of the reasons [38]. In addition, the role of HBsAg in the formation of immune tolerance in HBV infection should be considered [39, 40]. In order to investigate the effect that different HBsAg level has on induction of specific immune responses, the mice from ADY model were divided into low and high HBsAg level groups before therapy. HBV vaccine only induced sustained seroconversion in mice with HBsAg below 1000 COI, which confirmed that high HBV antigen level limited the immune response to therapeutic vaccine [40].

Activation of TLR7 could induce innate immune response with specific cytokines [26]. Here, we found that TLR7 agonist GS-9620 can inhibit the level of HBsAg. Interestingly, our mouse models with extremely low level of HBsAg (less than 100 COI) did not respond to the stimulation of GS-9620 (data not shown). So maybe combining a therapeutic hepatitis B vaccine with an agonist of TLR would be helpful for exploring functional cure for CHB.

In summary, this mouse model would be beneficial for exploring the new mechanism of HBV clearance and new treatments for chronic HBV infection.

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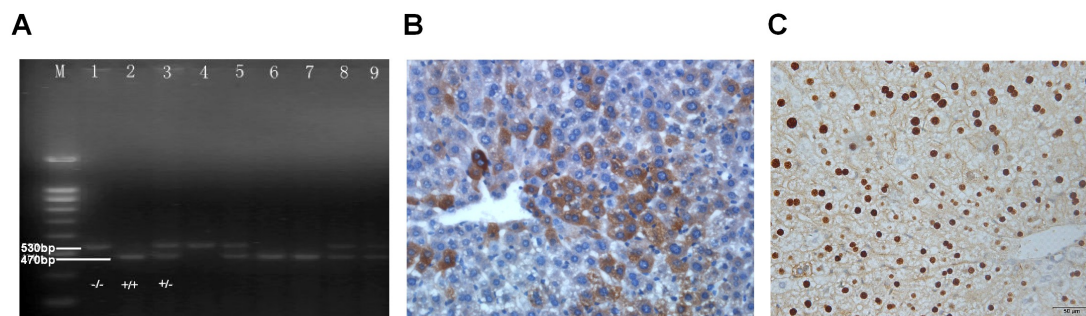


Fig.1. Genotype of HBVRag1 mice and expression of HBsAg, HBcAg in the liver (A). Total DNA of the mice was amplified by Rag-1-deficient mice gene sequence specific primers (P1、 P2 and P3). 530 bp fragment indicated that it was from Rag-1^{-/-} mouse, while 474 bp fragment was from Rag-1^{+/+} mouse, 530 and 474 bp fragments was from Rag-1^{+/-} mouse. Immunohistochemistry stained for HBsAg (B) and HBcAg (C) in liver.

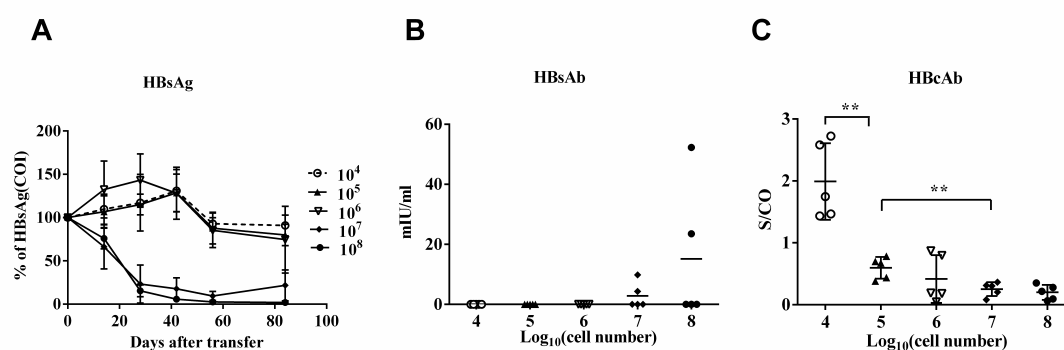


Fig.2. Number of adult splenocytes for adoptive transfer to HBVRag1 mice. After adoptive transfer the change of HBsAg (A) was determined during the following 12 weeks and the presence of HBsAb (B), and HBcAb (C) were determined at 4 weeks after the splenocytes transfer, S/CO < 1.0 was determined positive for HBcAb, n = 5 mice.

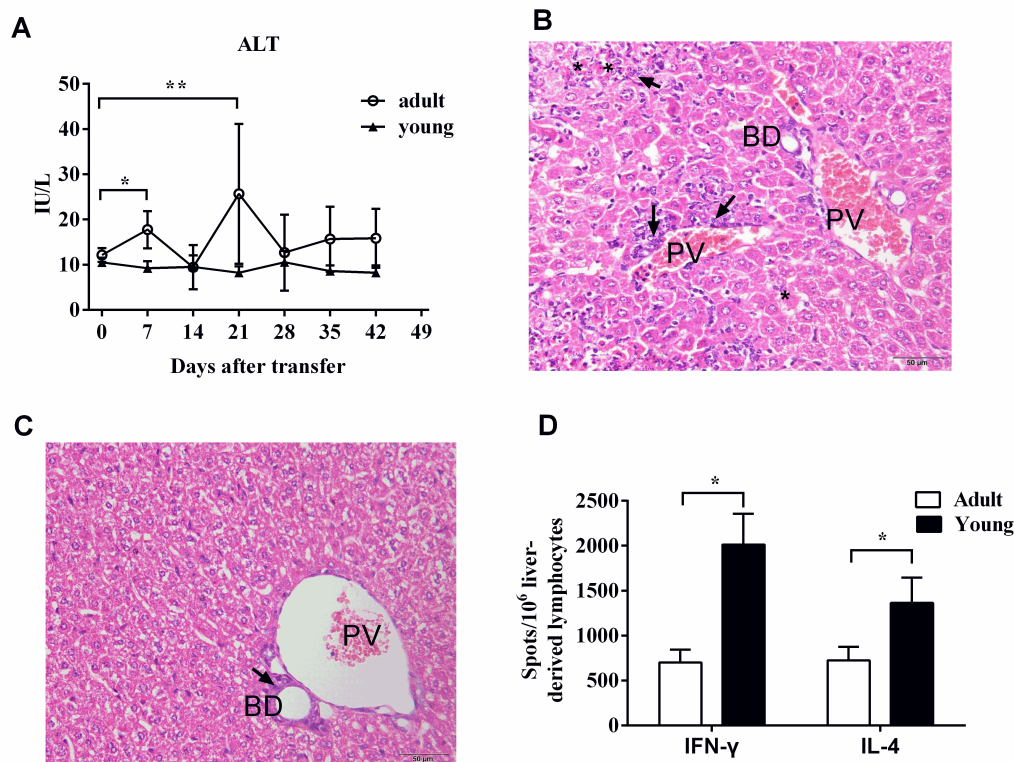


Fig.3. Adoptive transfer splenocytes leads to acute and chronic hepatitis in adult and young HBVRag1 mice respectively. Serum ALT from adult and young HBVRag1 mice after adoptive transfer splenocytes from adult BALB/c mice (A). Hepatic tissues from mice eight days after splenocytes transfer were stained with hematoxylin and eosin, adult (B) and young (C) HBVRag1 mice. Asterisks indicate necrotic hepatocytes. Arrowheads illustrate the inflammatory infiltrate surrounding portal vein (PV) or bile duct (BD). n = 6 mice. Results represent three independent experiments (E). IFN- γ and IL4 cytokines were detected by ELISpot assay of hepatic lymphocytes from adult and young HBVRag1 mice eight days after adoptive transfer. Cells were mixed from 4 mice.

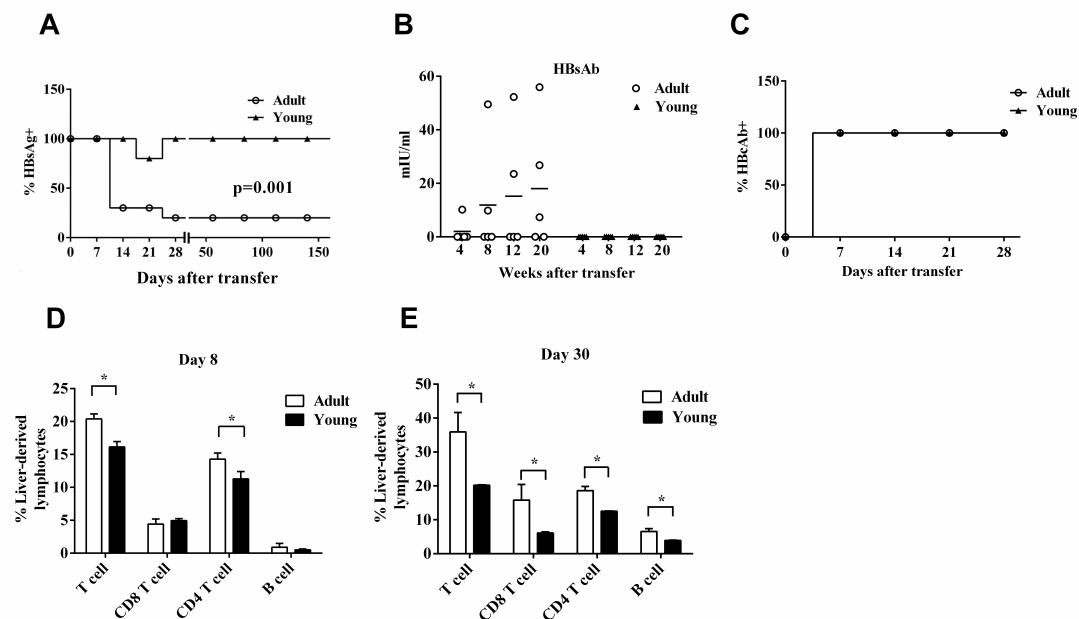


Fig.4. Difference in the clearance of HBsAg and production of anti-HBs, anti-HBc and dynamic changes of lymphocytes in the young and adult HBVRag1 mice livers after adult splenocytes transfer. The proportion of mice (% HBsAg⁺) with detectable HBsAg in the circulation (A) and serum HBsAb level (B), and the percentage of HBcAb positive mice (C) among young and adult HBVRag1 mice. Hepatic T and B cells in adult and young HBVRag1 mice 8 days (D) and 30 days (E) after adoptive transfer. CD4 T cell (CD3⁺CD4⁺), CD8 T cell (CD3⁺CD8⁺), B cell (B220⁺IgM⁺), $n \geq 5$ mice, Results represent at least two independent experiments.

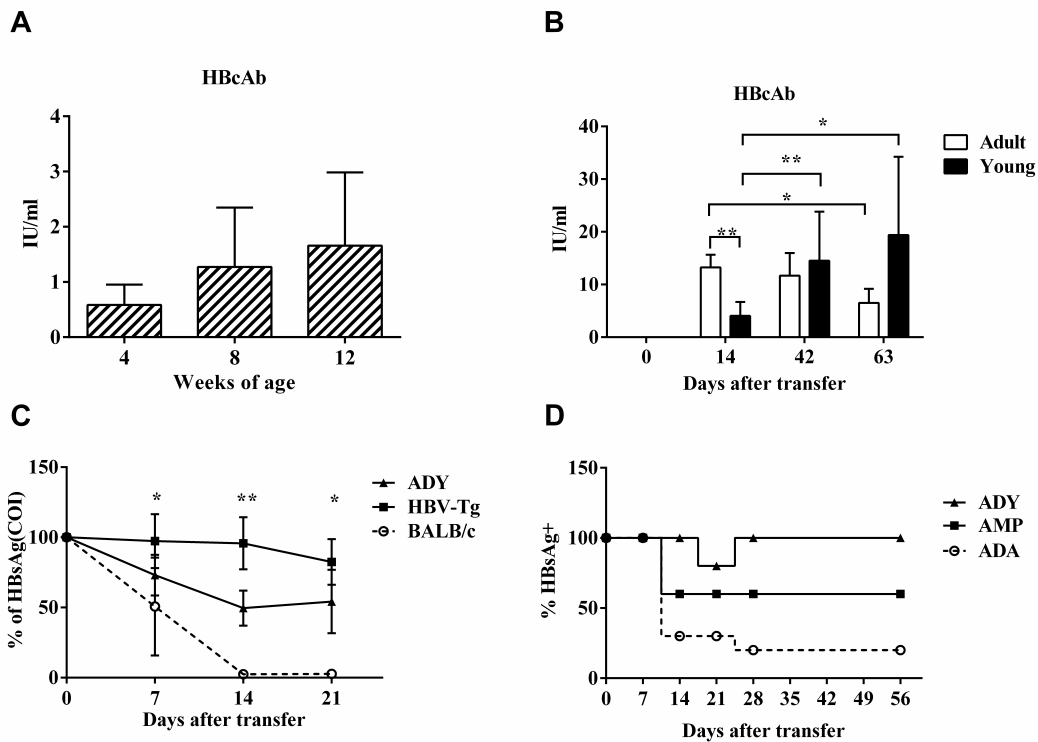


Fig.5. Age-related tolerance to HBV in ADY mouse model was different from HBV-Tg mice, and can be regulated by maturation of gut microbiota, and mimics kinetic change of age-dependent human HBV infection. The kinetic change of qHBcAb in HBV-Tg mice (A) and adult or young HBVRag1 mice after splenocytes transfer (B), $n \geq 4$. Adult HBVRag1 mice received spleen cells from ADY mouse model, HBV-Tg mice and BALB/c respectively (C). Serum HBsAg level was measured at multiple indicated time by ELISA assay ($n = 6$). After treating HBVRag1 mice with water contained ampicillin, the age-dependent HBV clearance disappeared in most of the splenocytes transferred adult mice (D), $n \geq 5$. Results represent at least two independent experiments, Error bars depict mean \pm SEM, * $P < 0.05$, ** $P < 0.01$.

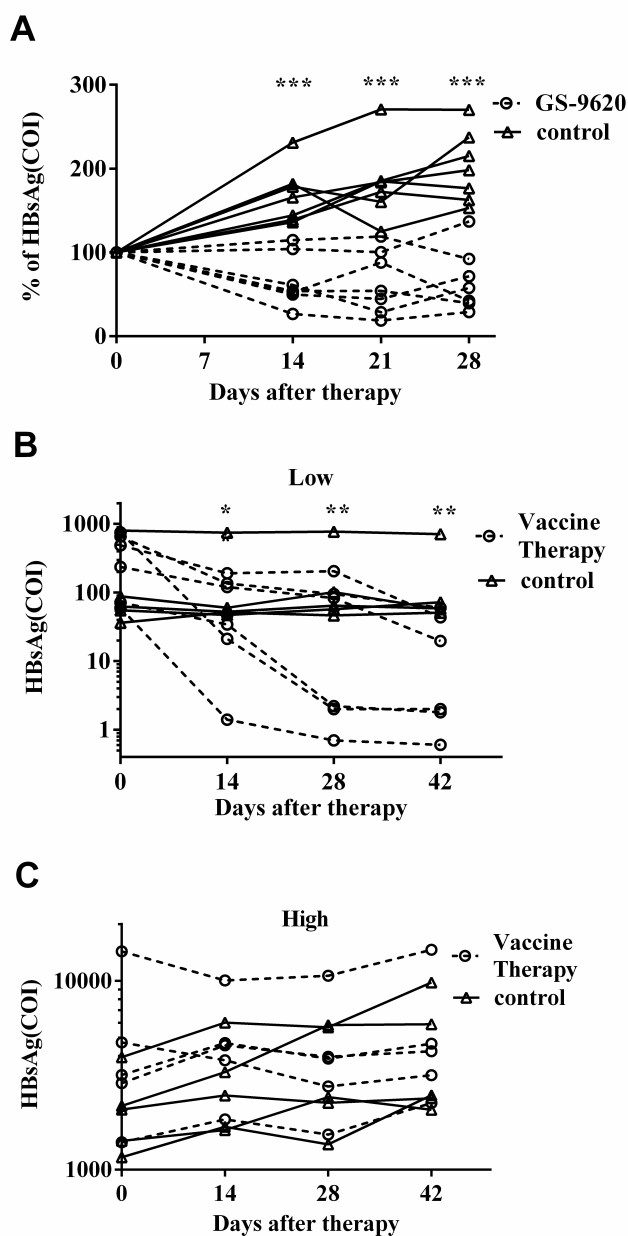


Fig.6. Anti-HBV activity of TLR7 agonist GS-9620 and HBV vaccine in the ADY mouse model. The ratio change of HBsAg (COI) compared to the beginning of treatment in ADY mice treated with GS-9620 (A). Shown are combined data from 2 independent experiments with mean \pm SEM (control, n = 7; GS-9620, n = 7). The changes of HBsAg (COI) after Hepatitis B vaccine immunization in low HBsAg level group (B) and in high HBsAg level group (C) within ADY mouse model. Shown are combined data from 2 independent experiments (control, n = 5; vaccine,

$n \geq 5$).